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Fourier transform infrared evidence for proline structural changes during the bacteriorhodopsin photocycle

(proton transport/purple membrane)

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ABSTRACT **Structural changes involving bacteriorhodopsin proline residues have been investigated by Fourier transform infrared difference spectroscopy. Bacteriorhodopsin (bR)-producing *Halobacteria halobium* were grown on a stringent medium containing either ring-perdeuterated proline or ^{15}N-labeled proline. Comparison of the difference spectra obtained from the photoreactions of these labeled bR samples with those for unlabeled bR has led to the assignment of peaks due to proline vibrations. [*proline*-N^{15}]bR exhibited a 15-cm^{-1} isotopic downshift of peaks in the 1420- to 1440-cm^{-1} region of the bR→K and bR→M difference spectra as well as a similar downshift of peaks found in the absolute absorption spectrum of bR. In contrast, [*proline*-D$_7$]bR did not cause shifts in this region of the difference spectra. These results indicate that one or more prolines undergo a structural rearrangement during the bR photocycle involving the Xaa-Pro C—N peptide bond. This change may be directly coupled to the light-induced isomerization of the retinal chromophore from all-*trans*-retinal to 13-*cis*-retinal.**

Bacteriorhodopsin (bR) is a M_r 26,000 integral membrane protein that functions as a light-driven proton pump in the purple membrane of *Halobacteria halobium* (1). A primary result of photon absorption by bR is the all-*trans*→13-*cis* isomerization of the retinylidene chromophore (2). This isomerization induces conformation and protonation changes in the protein that ultimately result in proton transport. While considerable information has been obtained from resonance Raman (3) and NMR spectroscopy (4) regarding the structural changes of the retinylidene chromophore during the bR photocycle, relatively little is known about the conformational changes that occur in the protein.

To probe the protein structural changes in bR, we have utilized Fourier transform infrared (FTIR) difference spectroscopy (5). This technique, when combined with isotopic labeling and site-directed mutagenesis, has the ability to provide information at the single group level (for a recent review, see ref. 6). Previously, several groups have used FTIR difference spectroscopy in conjunction with isotopic labeling to study the role of tyrosine (7, 8), aspartic acids (9, 10), and tryptophan (11) in the bR photocycle. FTIR difference spectroscopy in combination with site-directed mutagenesis has led to the identification of a tyrosine residue (Tyr-185), tryptophan residue (Trp-86), and aspartic residues (Asp-85, -96, -115, and -212) whose vibrations contribute to the FTIR difference spectra (12–14).

Recent interest has focused on the role of proline residues in bR as well as other membrane proteins. Integral membrane proteins involved in transport or that serve as receptors often contain proline residues in hydrophobic transmembrane segments of the amino acid sequence (15). It has been suggested that such proline residues play a role in transport mechanisms by undergoing structural alterations, including isomerization about the Xaa-Pro peptide bond (15) (where Xaa is an unspecified amino acid) or protonation at the imino nitrogen (16). Alternatively, proline may be more important as a structural determinant.

In this paper we report identification of shifts induced in the FTIR difference spectra and absolute absorption spectrum of bR because of labeling of bR proline residues. Contributions from the Xaa-Pro C—N peptide stretch are assigned on this basis in both the bR→K difference spectrum and bR→M difference spectrum. These results indicate that the Xaa-Pro C—N bond of one or more proline residues is structurally active during the bR photocycle.

MATERIALS AND METHODS

Preparation of Isotopically Labeled bR Samples. The JW-3 strain of *H. halobium* was grown in a synthetic medium like that of Gochnauer and Kushner (17), except that the D amino acids and the ammonium chloride were omitted, and the usual L-proline was replaced with 0.05 g of L-[D$_7$]proline (L-proline with the pyrrolidene ring perdeuterated) or 0.10 g of L-[^{15}N]proline per liter with a trace amount of L-[5-^{3}H]proline. Purple membranes were isolated by the procedure of Oesterhelt and Stoeckenius (18). Ammonia/acetone extraction showed that at least 95% of the incorporated radioactivity was in the protein, and of this, amino acid analysis showed that less than 5% was scrambled to other amino acids. Specific activity measurements indicated that nearly half of the proline residues were labeled with the perdeuterated L-[D$_7$]proline, and almost all of the proline residues were labeled with L-[^{15}N]proline.

FTIR Measurements. FTIR difference measurements were made as reported in detail elsewhere (19, 20) on rehydrated films formed by air-drying a purple membrane suspension on a AgCl window. The sample is sealed in a specially designed cell, light-adapted, and cooled to either 250 K (bR→M) or 77 K (bR→K) (20, 21). All measurements were made under steady-state illumination at 2-cm^{-1} resolution with a Nicolet 60SX spectrometer. The bR→K difference spectrum was obtained by accumulating 2000 interferograms under constant green illumination (500 nm) to produce the K intermediate, accumulating another 2000 interferograms under red illumination (650 nm) to photoreverse to the bR state, and then Fourier-transforming both signal-averaged interfero-

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Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; a.u., absorbance units.

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grams and subtracting the bR spectrum from the K spectrum. On each sample this cycle was repeated at least 50 times, and the differences were averaged. In the case of the bR→M difference spectra, a similar procedure was used with cycles of constant yellow illumination (550 nm) followed by no illumination. Highly reproducible difference spectra are obtained with this method for bacteriorhodopsin, rhodopsin, halorhodopsin, and the photosynthetic reaction center (6).

RESULTS

Absorption Spectrum of bR Containing Labeled Proline Residues. Fig. 1 compares the infrared absorption spectrum of light-adapted bR at 77 K with those of the two proline-labeled bR samples recorded under identical conditions in the 1400- to 1475-cm^{-1} region. In the case of the [*proline*-^{15}N]bR spectrum, the 1424-cm^{-1} band appears to shift down to 1410 cm^{-1}. However, a shift in the 1426-cm^{-1} band is not apparent for the [*proline*-D_7]bR spectrum. This indicates that the 1424-cm^{-1} band arises from a proline vibrational mode that involves the proline ring nitrogen but not the ring hydrogens. In contrast, [*proline*-D_7]bR exhibited an increased intensity near 1430 cm^{-1} and a small reduction in intensity at 1456 cm^{-1}. However, the 1424-cm^{-1} band is still present, although more apparent in the deconvolved spectra (data not shown). This indicates that a mode involving the ring hydrogens occurs near 1456 cm^{-1}. However, the relatively small isotope-induced shift indicates that this mode is not a pure C—H wag.

Infrared measurements on model compounds support our assignments. A strong band at 1430 cm^{-1} is observed in the spectrum of poly(L-proline) films (Y.-W.H. and K.J.R., unpublished data) that was previously assigned without the aid of isotopic labels to a C—H bending mode of the pyrrolidine ring (22). However, several studies based on isotopic labels indicate that the C—N stretch mode for disubstituted amides should also occur in this region (23, 24). We found that L-proline films had little absorption near 1430 cm^{-1}, which is consistent with the absence of an Xaa-Pro peptide bond (data not shown). A reduction in intensity of a band at 1450 cm^{-1} in the absorption spectrum of L-proline due to D_7-ring labeling but not to ^{15}N labeling of the proline peptide nitrogen supports the location of the C—H bending mode of the proline ring hydrogens nearer to this frequency.

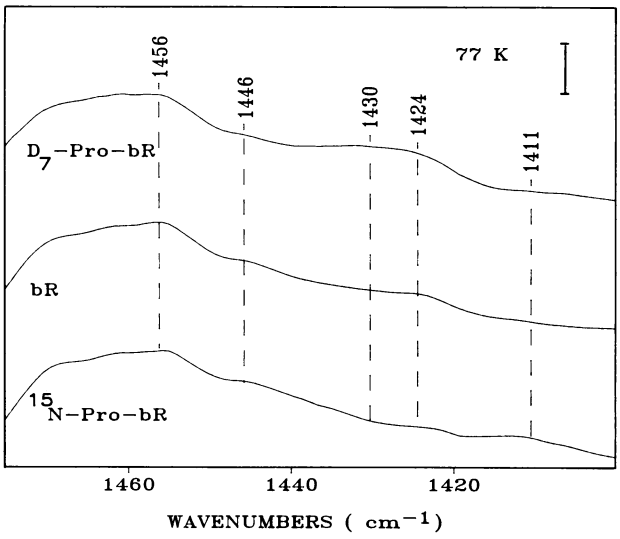

FIG. 1. Infrared absorption of bR (middle spectrum); bR labeled with L-[^{15}N]proline (bottom spectrum), and bR labeled with L-[D_7]proline (top spectrum). Spectra were recorded from light-adapted, humidified films cooled to 77 K and kept under constant red (650 nm) illumination to avoid production of the K intermediate. Individual spectra shown have no smoothing and consisted of over 50,000 coadded scans recorded at 2-cm^{-1} resolution, which resulted in a noise level in the region shown of $<10^{-4}$ absorbance units (a.u.). The scale marker corresponds to an adsorbance increment of 0.05 a.u. for the bR spectrum.

FTIR Difference Spectra. *bR→K photoreaction.* Fig. 2 compares the FTIR difference spectra of the bR→K transition for unlabeled bR and for bR containing labeled prolines. In normal bR, a pair of peaks is found at 1423 cm^{-1} (negative) and 1429 cm^{-1} (positive), which are reduced or absent in the [*proline*-^{15}N]bR sample. These peaks appear to shift down approximately 15 cm^{-1} in [*proline*-^{15}N]bR, giving rise to a negative/positive pair at 1410 and 1417 cm^{-1}. Since the 1410-cm^{-1} band is superimposed on a negative peak appearing at a similar frequency in normal bR, it may be located at a lower frequency.

In contrast, we observe little change in this region for [*proline*-D_7]bR, in agreement with the absence of a shift observed in this region for the absolute absorption of [*proline*-D_7]bR. Thus, we conclude that a structural alteration occurs during the bR→K transition that involves the Xaa-Pro C—N bond of one or more prolines. There is also a change near 1453 cm^{-1} in the case of the [*proline*-D_7]bR, which could be indicative of an alteration in the C—H bending mode of the proline ring.

bR→M photoreaction. The spectral shifts induced by isotopic labeling in the 1400- to 1475-cm^{-1} region of the bR→M difference spectra are similar although not identical to the bR→K results (Fig. 3). In the case of [*proline*-^{15}N]bR, the negative/positive pair near 1421/1431 cm^{-1} appears to shift to 1401/1416 cm^{-1}. In contrast, the [*proline*-D_7]bR spectrum exhibited little alteration in this region. Since the intensity of peaks in the 1420- to 1440-cm^{-1} region of the bR→K and bR→M difference spectra are similar, proline structural changes that occur by K may persist at M. Almost identical features are also observed in time-resolved FTIR difference spectrum of the bR→M transition at room temperature (25). Thus, the proline changes that we observe cannot be due to the effects of low temperature.

DISCUSSION

In this study, we have been able to assign peaks in the 1420- to 1440-cm^{-1} region of the bR→K and bR→M difference spectra to proline vibrations. On this basis, we conclude that

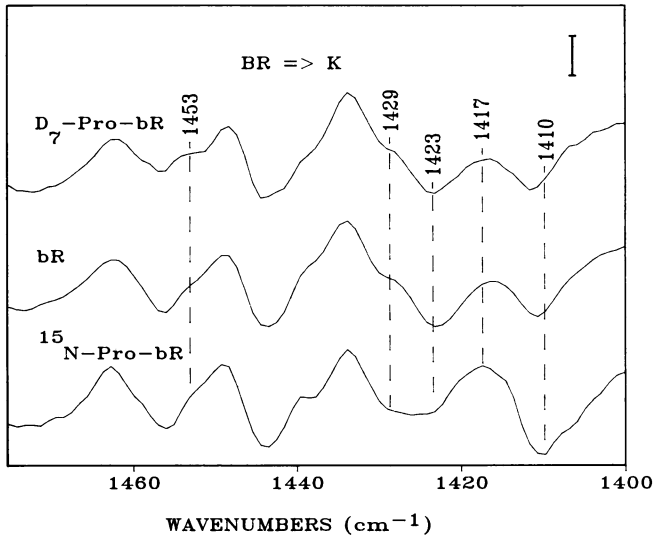

FIG. 2. bR→K difference spectra for [*proline*-D_7]bR (top spectrum), bR (middle spectrum), and [*proline*-^{15}N]bR (bottom spectrum) recorded at 77 K for light-adapted humidified films (see ref. 21 for further details). The scale marker corresponds to an absorbance increment of 0.001 a.u. for the bR spectrum.

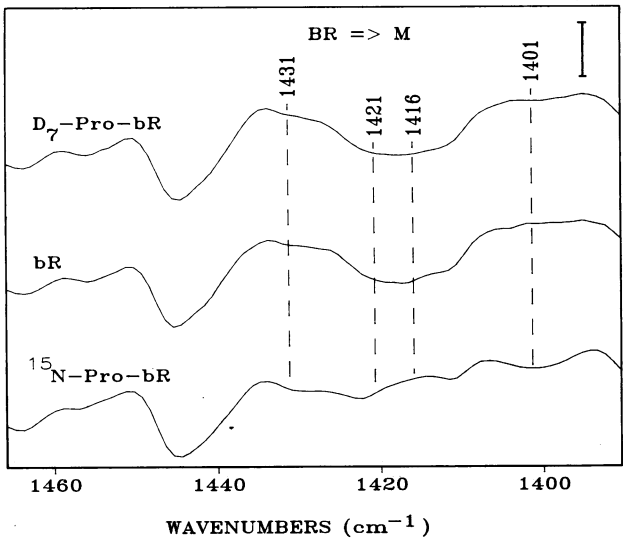

FIG. 3. bR→M difference spectra for [*proline*-D_7]bR (top spectrum), bR (middle spectrum), and [*proline*-^{15}N]bR (bottom spectrum) recorded at 250 K under steady-state illumination conditions for light-adapted humidified films (see ref. 19 for further details). The scale marker corresponds to an absorbance increment of 0.0005 a.u. for the bR spectrum.

at least one proline residue undergoes a change during the bR photocycle. At low-temperature, this change occurs by the primary photointermediate K, whose formation involves an all-*trans* to 13-*cis* isomerization of the retinylidene chromophore. Thus, it is likely that as a consequence of chromophore isomerization, one or more prolines are altered. The fact that similar spectral changes appear in the bR→M difference spectrum indicates that the proline change that occurs by K persists at least until the M intermediate.

The exact nature of the proline changes remains to be determined. The present study establishes that these changes produce an alteration in a mode that involves the Xaa-Pro C—N bond. The upshift in frequency of the 1424-cm^{-1} peak of light-adapted bR upon formation of K is similar to the shift of a similar band observed upon partial conversion of polyproline I to polyproline II films upon humidification (26). Since this transition may involve a cis-trans isomerization around the Xaa-Pro C—N bond, a similar isomerization of a proline in bR is possible. However, the shift we observe might also be induced by other perturbations of the proline, including charge perturbation near this bond.

In addition to proline, FTIR difference spectroscopy combined with site-directed mutagenesis and isotopic labeling has led thus far to the identification of several other amino acids that undergo a change during the primary phototransition. These include Tyr-185 (helix F) (12), Trp-86 (helix C) (14), and Asp-115 (helix D) (13). One simple explanation of these findings is that these are residues located close to the chromophore and are perturbed by chromophore isomerization. In this regard, a model of the bR retinal binding pocket has been proposed (14, 27) that predicts the presence of the residues Tyr-185, Trp-86, and Pro-186 in the retinal binding site. Thus, Pro-186 is a residue that might be perturbed upon chromophore isomerization.

Two other residues, Pro-91 (helix C) and Pro-50 (helix A), are also located in transmembrane regions of bR. Pro-91 is a particularly good candidate for a structurally active proline since it is located on helix C. This helix includes three other residues, Asp-85, Asp-96, and Trp-86, that appear to undergo structural alterations during the photocycle (13, 14). However, none of the buried prolines (Pro-50, -91, and -186) appear to be necessary for proton pumping (28). Therefore, the remaining eight proline residues should also be considered possible candidates for the active proline(s) detected here. The surface prolines are found mainly in linking regions between the transmembrane helices and might facilitate movement of these helices relative to each other by altering the Xaa-Pro peptide bonds.

CONCLUSIONS

Our results establish that one or more prolines are active during the bR photocycle. These changes appear to involve the Xaa-Pro C—N peptide bond, although it is not clear whether this reflects a cis→trans isomerization about the bond. Furthermore, this work does not establish which proline residue(s) are involved. Studies combining isotopic labels, site-directed mutagenesis and FTIR can provide further information concerning this question (K.J.R., Y.-W.H., J.H., R.S.B., T. Mogi, T. Marti, and H. G. Khorana, unpublished data).

Our findings also have significance beyond bR. In particular, it has been suggested that buried prolines play a role in the functioning of other membrane proteins. For example, in the case of the related rhodopsin proteins, there exist three highly conserved buried prolines (29), one of which occurs in a putative retinal binding pocket (27). A recent study of the *lac* permease from *Escherichia coli* (30) indicates that buried prolines can usually be replaced by alanine with near normal transport, while the bulkier residue leucine interfers with transport. In general, it is not clear from these findings if buried prolines are structurally active or required for proper protein folding. A similar approach, which combines FTIR and isotopic labeling, may be useful for investigating the role of prolines in these membrane proteins.

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